APPENDIX B

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Relationship between salivary melatonin levels and periodontal status in diabetic patients

Abstract: Among other functions, melatonin exerts both antioxidative and immunoregulatory roles. The indoleamine is secreted in the saliva, although its role into the mouth is not known. Diabetic patients frequently display oral cavity pathologies such as periodontal disease (PD), an inflammatory disease coursing with an increase in free radical production. Thus, we compared the degree of PD and interleukin-2 (IL-2) levels with melatonin concentrations in plasma and saliva of diabetic patients. A total of 43 diabetic patients (20 with type I and 23 with type II diabetes) and 20 age- and sex-matched controls were studied. Dental and medical history of all patients was in accordance with the criteria of the WHO. The periodontal status was evaluated by the Community Periodontal Index (CPI). Plasma and salivary melatonin levels were determined by specific commercial radioimmunoassays, and plasma IL-2 was measured using a commercial enzyme-linked immunosorbent assay kit. Diabetic patients had plasma and saliva melatonin levels of 8.98 ± 7.14 and 2.70 ± 2.04 pg/mL, respectively. These values were significantly lower ($P < 0.001$) than those obtained in plasma and saliva of controls (14.91 ± 4.75 and 4.35 ± 0.98 pg/mL, respectively). Plasma and salivary melatonin concentrations show a biphasic response in diabetic patients. Melatonin decreased in patients with a CPI index of 2, and then increased reaching highest levels in patients with a CPI index of 4. By contrast, IL-2 levels decreased from CPI index 1 to 4. The results indicate that, in diabetic patients, the presence of a marked impairment of the oral status, as assessed by the CPI index, is accompanied by an increase in plasma and salivary melatonin. The increase in salivary melatonin excretion may have a periodontal protective role.

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Key words: community periodontal index, diabetes, interleukin-2, melatonin, saliva

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Introduction

Periodontal disease (PD) is an oral inflammatory process affecting the alveolar bone, gums, and periodontal ligament. The ethiopathogeny and pathophysiology of the PD is not clear. The presence of microorganisms in the oral cavity initiates a series of processes leading to the damage of healthy tissues. The damage of periodontal tissues results from a direct effect of the toxic products released by the bacteria, and from the action of the immune system stimulated by the bacterial infection [1]. Nevertheless, an important feature in PD is the generation of free radicals, some of which derive from the bacteria themselves, and others originate from the immune response [2]. It is suggested that an increase in both reactive oxygen and nitrogen species during PD is responsible for the oxidative damage to periodontal tissues [3]. The increase in free radical production coexists with a decrease in the antioxidant defense. The imbalance between the prooxidant and antioxidant systems may lead to a further oxidative attack and substantial deterioration of the periodontal tissues [4, 5].

Melatonin is a noteworthy free radical scavenger and a broad-spectrum antioxidant [6–9]. In certain pathologies associated with oxidative stress, melatonin displays both anti-inflammatory and bone repair effects [10–13]. About 24–33% of the plasma melatonin appears in the saliva, where it is easily measured by radioimmunoassay (RIA) [14, 15]. Salivary melatonin measurement provides a readily accessible means of obtaining data on the melatonin excretion via this route. A significant positive correlation between salivary and plasma melatonin exists and the former is a reliable predictor of plasma melatonin levels. By measuring salivary melatonin, oral pathologies can be studied in relation to plasma and salivary melatonin behavior.

In response to antigenic stimuli, T lymphocytes produce interleukin-2 (IL-2). IL-2 regulates a series of processes in different cells of the immune system including natural killer cells, monocytes/macrophages and B lymphocytes. A relationship between IL-2 and melatonin was described when it was found that melatonin stimulates the production of IL-2 by T lymphocytes [16]. It is known that the metabolic products of periodontopathic bacteria decrease cytokine production including IL-2 [17, 18]. Thus, it was of interest

to study the changes in melatonin, IL-2 relationships during periodontal pathologies.

Accordingly, the objective of this study was to assess plasma IL-2 levels and correlate them with plasma and salivary melatonin levels in patients with type I, (DM-I), and type II, (DM-II), with different degrees of PD according to the Community Periodontal Index (CPI) [19]. These data were compared with those measured in a control group of healthy subjects to assess any change in melatonin levels that may be related to the periodontal status and/or immune response.

Materials and methods

Patients

The study was carried out in the Faculty of Odontology (Granada, Spain). A total of 63 patients were included in the study. Information was given and authorization obtained from the patients and from the University's Ethical Committee, and the Code of Ethics of the World Medical Association was observed. Subjects were classified in two groups: a control group that comprised 20 healthy subjects (12 women and eight men), aged 48.9 ± 10.3 yr. The subjects of this group were age- and weight-matched with patients in the diabetic individuals, comprised 43 patients (20 with DM-I and 23 with DM-II). Dental and medical history of all patients was in accordance with the criteria of the WHO. The periodontal status was evaluated by the CPI.

The inclusion criteria for DM patients were: (a) age between 18 and 65 yr old, and (b) glycosylated hemoglobin between 7.6 and 8.0% during the last 6 months, values compatible with a tolerable control of diabetes. Exclusion criteria included the presence of other concomitant systemic pathologies such as epilepsy and schizophrenia, and diseases that may affect the immune system such as chronic infectious and oncological diseases [20, 21]. Patients under pharmacologic treatment that could alter melatonin levels were also excluded from the study. The clinical history also included a dental history following the simplified WHO criteria [19], including both dental (presence or absence of caries) and periodontal (CPI index) status. The oral cavity explorations and clinical data were always analyzed by the same person. A concordant diagnostic analysis was performed, in which other odontologists carried out the oral exploration in 11 cases. This inter-observer assay yielded a concordance coefficient of 81% for the CPI index.

The CPI index is currently recommended by WHO, and it consists of dividing the oral cavity in to six sextant with index teeth in each one. Teeth index are 17/16 for the first sextant, 11 for the second, 26/27 for the third, 36/37 for the fourth, 31 for the fifth, and 47/46 for the sixth. Teeth are examined with a probe with two marks at 8.5 and 11.5 mm, which permits the measurement of the dental bone affectation's degree by the inflammatory process. The CPI codes used for recording the periodontal status are the following: code 0, healthy periodont; code 1, moderate bleeding; code 2, presence of supra- or sub-gingival dental calculus; code 3, periodontal sac of 4–5 mm; and code 4, periodontal sac of 6 mm or higher.

Plasma melatonin determination

Patients came to the laboratory at 08:00 hr, and they were seated 30 min before they were sampled. Blood samples were taken from the antecubital vein and centrifuged at 3000 g for 10 min, and plasma was separated and frozen at −20°C until further use. Plasma melatonin was determined by a commercial RIA (DVD BIOCHEMIE, Marburg GmbH, Germany) and a quality control was performed showing an intra- and inter-assay coefficients of variation of 11.3 and 6.3%, respectively. The recovery of added melatonin was 84.4% and the sensitivity of the assay was 4.65 pg/mL.

Salivary melatonin determination

Saliva was obtained after chewing a piece of paraffin. Saliva produced during the first 2 min was discarded. Then, saliva was collected during the following 5 min, avoiding any possible contamination. The patients chewed the paraffin during the time of saliva collection [22]. Samples of collected saliva were centrifuged at 3000 g for 20 min, and the clear supernatant was frozen to −20°C. Melatonin levels in saliva were measured by RIA (IBL, Hamburg GmbH, Germany) [23]. The quality control of melatonin RIA showed an intra- and inter-assay coefficients of variation of 12.9 and 7.2%, respectively. The recovery of added melatonin was 80.3% and the sensitivity of the assay was 2.19 pg/mL.

Plasma IL-2 determination

Plasma IL-2 was determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Chemicon International, Temecula, CA, USA). The sensitivity of the method was determined by assaying replicates of zero and the standard curve. The mean signal of zero + 2 S.D.s read in dose from the standard curve is the lower limit of detection. This value is the smallest dose that is not zero with 95% confidence. The standards in the ELISA were calibrated to the National Institute for Biological Standards and Control (NIBSC) reference lot 86/504. One picogram of endogen standard = 0.77 NIBSC units. The intra- and inter-assay coefficients of variation were < 10% in each case.

Statistical analyses

Quantitative variables are expressed as the mean ± S.D., whereas absolute and relative frequencies were calculated for qualitative variables. To correlate quantitative with qualitative variables, Mann–Whitney test was used. Student's *t*-test was used to compare the mean values of quantitative variables, and the Spearman's correlation coefficient was used to correlate quantitative variables with qualitative ones. The relationship between one qualitative variable with more than two modalities (CPI index) and the quantitative variables was examined by the Kruskal–Wallis test an/or one-way ANOVA.

Results

Table 1 shows the data corresponding to the control and diabetic subjects. Diabetic patients had significant lower

Table 1. Comparison between the studied variables in controls and diabetic patients

Variable	Controls (n = 20)	Diabetics (n = 43)
Age	48.9 ± 10.3	53.8 ± 12.4
PM	14.91 ± 4.75	8.98 ± 7.14**
SM	4.35 ± 0.98	2.70 ± 2.04**
SM/PM	0.30 ± 0.06	0.31 ± 0.07
IL-2	4.00 ± 0.99	4.10 ± 2.07

Data are expressed as mean ± S.D. Age is expressed in years and melatonin and IL-2 in pg/mL. n, number of cases. **$P < 0.001$ versus control.
PM, plasma melatonin; SM, saliva melatonin.

Table 2. Mean age of diabetic patients according to the CPI index

CPI index	n	Age (yr)
1	9	41.6 ± 11.5
2	10	51.9 ± 9.5
3	20	58.3 ± 11.2
4	4	63.3 ± 8.7

Data are expressed as mean ± S.D. n, number of cases.

levels of plasma and salivary melatonin than the corresponding healthy subjects ($P < 0.001$). Interestingly, the salivary/plasma melatonin ratio was similar in both groups.

A significant negative correlation was found between plasma melatonin and age in the control group ($r = -0.681$, $P < 0.001$). Interestingly, this correlation disappeared in the diabetic group ($r = 0.02$, NS).

Table 2 shows the CPI index according to the age of the diabetic patients. Application of Kruskal–Wallis test displayed a $\chi^2 = 17.78$ ($P < 0.001$), reflecting the age-dependent increase of CPI index.

We next asked if the diabetes type, DM-I and DM-II, might influence the studied variables. For this purpose, plasma and salivary melatonin and plasma IL-2 levels were studied in relation to the type of diabetes. Fig. 1 shows that, although patients with type II diabetes had higher plasma and saliva melatonin levels than those with diabetes type I, these changes were not significant. IL-2 was also unchanged by the type of diabetes (Fig. 1).

A biphasic relation between plasma melatonin and CPI index was found (Fig. 2). Plasma melatonin decreased in patients with CPI index of 1 (youngest patients) to CPI 2, and then increased reaching highest values at CPI 4. Salivary melatonin parallels the changes of plasma melatonin. IL-2 behaved differently than melatonin (Fig. 2). Highest concentrations of IL-2 were found in diabetic patients with the lowest CPI index. When the CPI index increased, IL-2 decreased, reaching the lowest levels at CPI index of 3. It is of interest to note that IL-2 seems to increase at CPI index of 4, although these changes were not significant.

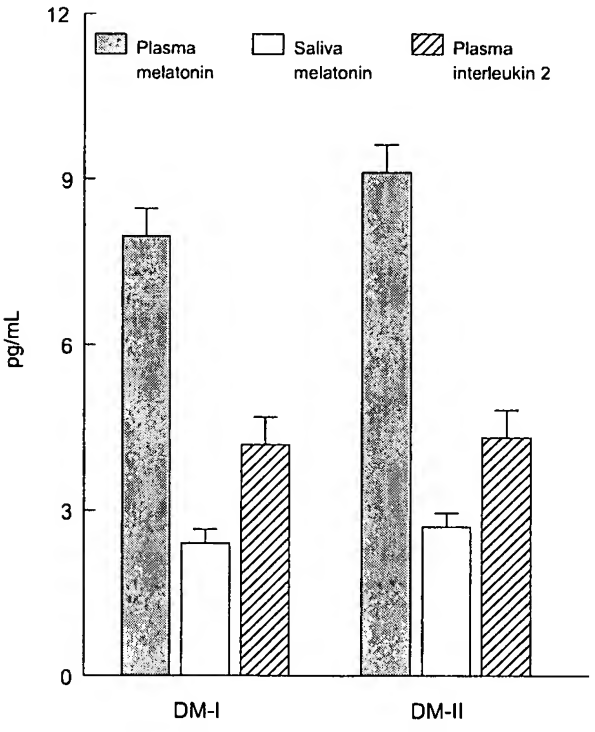

Fig. 1. Plasma and saliva melatonin and IL-2 levels in relation to the diabetes type. Although plasma and saliva melatonin and IL-2 leves were higher in DM-II patients, only the former showed significant changes ($P < 0.005$).

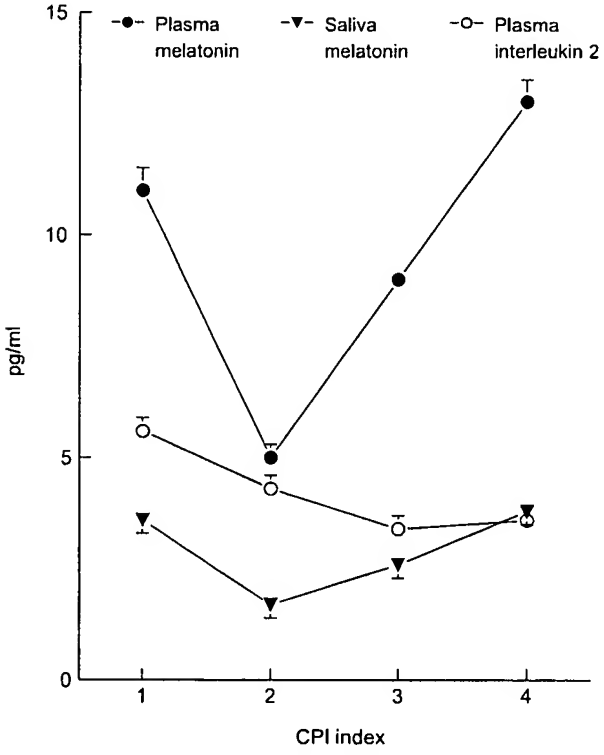

Fig. 2. Plasma and saliva melatonin and IL-2 levels in diabetic patients in relation to the CPI index. A transient decrease in plasma and saliva melatonin levels was found at a CPI index of 2. IL-2 decreased significantly from a CPI index 1 to 3 ($P < 0.05$).

Discussion

The current results show that either in control group or in diabetic patients, melatonin excretion in saliva corresponds to 30–31% of plasma melatonin, which is in agreement with similar values previously reported in humans [24, 25]. The percentage of melatonin extraction in saliva is independent of the plasma melatonin concentration. However, the absolute concentration of salivary melatonin changes as a function of its plasma levels. Consequently, inflammatory processes such as periodontitis, which trigger signals to increase plasma melatonin concentration, also increase melatonin levels in the oral cavity, where the indoleamine may exert a protective role. Salivary melatonin determinations seem valid for studies on melatonin behavior in oral pathologies, and yield reliable data with a non-invasive procedure, which is very useful for the odontologist [26, 27].

According to the CPI index, the periodontal status deteriorates with age. Melatonin levels significantly decreased from CPI index 1 to 2, which may be related to the age-dependent reduction in both melatonin production and salivary levels [28, 29]. An interesting finding in this study is that, when the CPI index is 3–4, corresponding to a serious periodontal process with bone damage and gingival involvement, melatonin levels increase. Thus, patients with higher CPI indexes, and thus older than those with lower CPI index, have high melatonin levels. Since higher CPI indexes correspond to a worst periodontal status, the melatonin increase may be a consequence of a signal(s) derived from the oral inflammatory process [30, 31]. It is suggested that the organism responds to a periodontal inflammation by increasing melatonin production and thus, melatonin availability to the oral cavity. Melatonin may participate in the restoration of alveolar bone stimulating the proliferation of type I collagen cells [32] and modulating both osteoclastic and osteoblastic activities [12, 13, 33, 34].

It was shown that lymphocytes of patients with DM-I produce low IL-2 [35]. However, no correlation between IL-2 and periodontal status was investigated in these reports. Our results show that IL-2 levels are unrelated to the type of diabetes but they significantly change with the periodontal status reaching lowest levels at highest CPI indices. Perhaps the significant melatonin increase at CPI 4 may be somewhat responsible for the slight elevation in IL-2 [16, 36]. Patients with DM-I show a lesser response in both melatonin and IL-2, suggesting that the immunostimulating and anti-inflammatory properties of melatonin are also depressed in these patients. It is important to note that in these patients, periodontal processes yield premature involvement of both alveolar and dental bone [10, 11, 37–39].

Previous data have shown the existence of an inverse relationship between peroxidation products and the quantity of antioxidants in periodontal pathology [40]. A key finding in periodontitis is polymorphonuclear neutrophils infiltration; these cells produce high amounts of reactive oxygen species (ROS). Moreover, a massive neutrophil migration to the gingiva and gingival fluid during periodontitis leads to abnormal spreading of ROS [41]. At least part of the antioxidant potential in the oral cavity relates to uric acid and, to a lesser extent, to vitamin C and albumin [42, 43]. No clear evidence has emerged in relation to the possible antioxidant activity of vitamin A or CoQ in periodontitis. Some studies show that a deficiency in vitamin E, another antioxidant, does not increase in periodontitis; in general, studies do not provide any support for the treatment of periodontitis with vitamin E. An interesting observation is that in older subjects more affected by periodontitis, vitamin E tended to increase [41]. Our results show a significant increase in melatonin levels in older patients having more periodontal damage. Thus, the melatonin rise in PD may also be secondary to the increase in the free radical production in this pathology. Due to the antioxidant [6–9, 44, 45] and anti-inflammatory [46] effects of melatonin, the increase in salivary melatonin levels may improve the organism's response against the periodontal inflammatory process. These data agree with a role of melatonin against diabetes-induced oxidative stress [47, 48] and suggest that melatonin may participate in the antioxidative defense against free radical attack into the oral cavity.

From the above data, at least three important actions of melatonin, i.e. immunoenhancing, fibroblast proliferation and bone remodeling, and antioxidant, may be related to the presence of melatonin into the mouth. The importance of melatonin as an antioxidant in the oral cavity depends on its parallel effect on the immune system, which differs from other antioxidants such as vitamins A, E and CoQ, although the latter has a minor immunostimulatory role [49]. Also, it has been repeatedly shown that melatonin exerts a protective role against free radical damage during diabetes [50]. Thus, a melatonin increase during diabetes could protect all organs from oxidative damage, but this may be of particular importance into the mouth. Further studies on the melatonin effects into the oral cavity including its oxidative metabolites, would help to clarify the role(s) of melatonin into the mouth and the potential of its use in oral hygiene.

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Local Application of Melatonin Into Alveolar Sockets of Beagle Dogs Reduces Tooth Removal–Induced Oxidative Stress

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Background: The antioxidant and anti-inflammatory hormone melatonin is secreted by saliva into the oral cavity, where it may protect the mucosal and gingival tissues from radical damage. To date, no studies have addressed the potential beneficial role of melatonin in the acute inflammatory response that follows oral surgical interventions, especially tooth extractions. The aim of this study was to determine whether tooth extraction induces changes in plasma oxidative stress levels, and whether melatonin treatment may counteract these changes.

Methods: Maxillary and mandibular premolars and molars of 16 adult Beagle dogs were extracted under general anesthesia. Eight dogs were treated with 2 mg melatonin placed into the alveolar sockets, whereas the other eight dogs received only vehicle. Lipid peroxidation (LPO) and nitrite plus nitrate (NOx) levels were determined in plasma, whereas glutathione (GSH) and glutathione disulfide (GSSG) levels and glutathione peroxidase (GPx) and reductase (GRd) activities were measured in red blood cells before and 24 hours after tooth extraction.

Results: Removal of the premolars and molars caused a significant rise in plasma LPO and NOx levels and in the erythrocyte GSSG/GSH ratio, whereas melatonin treatment restored the normal values of these parameters. Also, melatonin slightly increased erythrocyte GRd activity without changing GPx activity.

Conclusion: For the first time to our knowledge, the results show that during the immediate postoperative period following tooth extraction, there is a significant increase of oxidative stress, which is counteracted by the administration of melatonin into the alveolar sockets. *J Periodontol 2007;78:576-583.*

KEY WORDS

Antioxidant; free radicals; mouth; oral surgery; oxidative stress.

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Reactive oxygen species (ROS), including superoxide anion radical, hydrogen peroxide, and the hydroxyl radical, and reactive nitrogen species (RNS), including nitric oxide (NO) and the peroxynitrite anion, are common byproducts produced by normal aerobic metabolism of oral cavity cells or by inhalation of oxidizing agents in tobacco smoke and other air pollutants.[1-4] Moreover, activation of the immune system by inflammatory processes, such as chronic periodontitis, increases ROS-RNS generation.[3] Although ROS are necessary for defense of the host, they also expose oral tissues to oxidative damage.[2] The mucosal barrier is the first line of defense against flora growing in the oral cavity. In mucosal cells, the production of NO by the expression of inducible NO synthase (iNOS) serves as a chemical barrier to limit bacterial plaque invasion. However, iNOS expression by oral epithelial cells is associated with diminished cell viability, which may depend on the peroxynitrite formation.[4,5] These reactive species are involved in the pathogenesis of several oral processes, including recurrent aphthous ulceration,[6] leukoplakia,[7] lichen planus,[8] and especially in oral cavity cancer and periodontal inflammatory disease.[9-12]

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Periodontal disease in its destructive phase is considered to be initiated and perpetuated by Gram-negative bacteria that colonize the subgingival area. When stimulated by periodontal pathogens, host cells release proinflammatory cytokines, whereas massive polymorphonuclear cell migration to the gingival crevicular fluid leads to abnormal spreading of ROS.[13,14] Additionally, macrophage infiltration to the periodontal tissues increases iNOS and NO, with the latter being related to the pathogenesis of periodontitis and subsequent bone loss.[4,15] Usually, an increase in free radical production coexists with a decrease in the antioxidant defense system.[6] The imbalance between the prooxidant and antioxidant systems may lead to further oxidative damage of periodontal tissues.[2,16-18]

Previous studies reported that oral inflammatory processes, such as periodontitis, can trigger signals that increase not only plasma melatonin (aMT) levels but also aMT levels in the oral cavity, where the indolamine may exert an antioxidant role.[19,20] The direct action of aMT as a free radical scavenger of both ROS and RNS[19,21,22] is complemented with an indirect stimulatory effect of the antioxidant enzymes, including glutathione peroxidase (GPx) and reductase (GRd), superoxide dismutase, and catalase.[23-25] Due to its stimulatory effect on GRd, aMT favors the recycling of glutathione (GSH) from glutathione disulfide (GSSG), maintaining a high GSH/GSSG ratio.[26] aMT also promotes the de novo synthesis of GSH by promoting the activity of γ-glutamyl-cysteine synthetase.[27] Additionally, aMT is also capable of reducing NO and peroxynitrite generation because of its ability to inhibit iNOS activity and expression, which increase the tissue damage that accompanies inflammation.[28,29] aMT also may exert an immunoenhancing role in the oral cavity because patients with severe periodontal status with bone damage and gingival involvement show concomitant high interleukin-2 and aMT levels, which may stimulate CD4 lymphocytes in response to periodontal disease.[30,31]

It is now well established that the acute inflammatory response of gingival tissue during the first 24 to 48 hours postextraction causes an important polymorphonuclear leukocyte infiltration, which is responsible, in part, for the increase in ROS and RNS generation.[32] Thus, the aim of this study was to test whether plasma changes reflect the acute inflammatory response caused by tooth extractions in Beagle dogs, and whether aMT treatment could modify any observed changes.

MATERIALS AND METHODS

Chemicals

GSH, GSSG, GRd, nicotinamide adenine dinucleotide phosphate (NADPH), cumene hydroperoxide, ophthalaldehyde, *N*-ethylmaleimide, methanol, *N*-(1-naphthyl) ethylenediamine dihydrochloride, sulphanilamide, trichloroacetic acid, and phosphoric acid were purchased,‖ and aMT was obtained.¶ All other reagents were of the highest purity available.

Animals, Surgery, and Treatment

The study was performed in 16 male Beagle dogs obtained from the Veterinary Faculty, University of Córdoba, Córdoba, Spain. The animals were maintained in the University's facility in individual kennels in a 12:12 light-dark cycle (lights on at 7:00 am) at 22°C ± 2°C with regular chow and tap water. Animals were 14 months of age at the time of the study and weighed 16 to 18 kg. All experiments were approved and performed according to the Spanish Government Guide and the European Community Guide for animal care.

Both upper and lower maxillary and mandibular premolars and molars of the 16 Beagle dogs were extracted under general anesthesia. The anterior group of teeth was conserved so that the dogs could maintain an appropriate masticatory function. All interventions were supervised by the veterinarian of the Animal Experimentation Service of the University of Granada. Fifteen minutes before general anesthesia, the animals received an intramuscular injection of 0.5 to 1 mg/kg acepromazine maleate, an anxiolytic. General anesthesia included ketamine plus chlorbutol, 5 to 8 mg/kg intravenously; 0.5 to 1 mg/kg acepromazine maleate as coadjuvant; and 0.05 mg/kg atropine. Dexamethasone isonicotinate (2 ml intramuscularly) and amoxicillin (2 ml intramuscularly) were administered at the end of surgery and every 2 days for a total of 4 days.

After the tooth extractions and before suturing, eight dogs received aMT applied into the extraction wounds and gingival tissue surrounding the premolar and molar area. The following groups of dogs were included: 1) control group (Con), consisting of all 16 dogs sampled 1 hour before tooth extractions; 2) vehicle-treated group (Veh), consisting of eight dogs with tooth extractions but without postextraction treatment; and 3) aMT-treated group (aMT), consisting of eight dogs receiving 2 mg powder aMT into the alveolar sockets and surrounding gingival tissue after surgical removal of the tooth.

Blood samples were taken from the vena cephalica antebrachii 1 hour before tooth extraction (control samples) and 24 hours after the surgical procedure. Blood was rapidly transferred to cold EDTA-K–containing tubes and centrifuged at 3,000 × g for 10 minutes at 4°C. Plasma aliquots were stored at –80°C for lipid peroxidation (LPO) and nitrite plus nitrate (NOx) determination. GSH and GSSG levels and GPx and GRd activities were determined in red blood cells.

‖ Sigma-Aldrich, Madrid, Spain.
¶ Helssin Chemicals, Biusca, Switzerland.

The cells were separated from the plasma and washed two times with 0.9% sodium chloride solution. Red blood cell aliquots were stored at –80°C until assays were performed.

LPO Determination

Malonaldehyde and 4-hydroxyalkenals concentrations provide a convenient index of lipid peroxidation. These lipid peroxidation products were determined with a special kit.# The kit takes advantage of a chromogenic reagent that reacts with malonaldehyde and 4-hydroxyalkenal (4HDA) at 45°C yielding a stable chromophore with maximal absorbance at the 586-nm wavelength.[33] Plasma LPO levels were expressed in nanomoles per milliliter.

NOx Determination

Levels of NOx were measured in plasma previously treated with nitrate reductase. Then, pretreated plasma aliquots were incubated with 100 µl of Griess reagent (0.1% N-[1-naphthyl] ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1) at room temperature for 20 minutes.[34] The absorbance at 550 nm was measured with a spectrophotometer.** NOx concentrations were calculated by comparison to the absorbance of a standard solution of known sodium nitrite concentration and expressed in nanomoles per milliliter.

Measurement of GSH and GSSG

Both GSH and GSSG were measured by a fluorometric method,[35] which was slightly modified. Aliquots of saline-washed red blood cells were thawed and hemolized (1:20) with 10 mM phosphate buffer, 1 mM EDTA-Na_2, pH 6.5, at 4°C for 5 minutes. Trichloroacetic acid was added at a final concentration of 5%, and the samples were centrifuged at 20,000 × g for 15 minutes at 4°C. For GSH measurement, aliquots of 10 µl of the trichloroacetic acid supernatants were incubated with 10 µl ophthalaldehyde solution (1 mg/ml in ethanol) and 180 µl phosphate buffer (100 mM sodium phosphate; 2.5 mM EDTA-Na_2; pH 8.0) for 15 minutes at room temperature. The fluorescence of the samples was measured in a plate-reader spectrofluorometer.†† A standard curve of known GSH concentrations was prepared and processed with the samples. For determination of GSSG levels, aliquots of 25 µl trichloroacetic acid supernatants were preincubated with 10 µl *N*-ethylmaleimide solution (5 mg/ml in distilled water) for 40 minutes at room temperature and alkalinized with NaOH 0.1 N. Aliquots of 10 µl were incubated with 10 µl ophthalaldehyde solution and 180 µl NaOH 0.1 for 15 minutes at room temperature. The fluorescence was measured, and GSSG concentrations were calculated according to a standard curve prepared accordingly. Hemoglobin (Hb) content in the red blood cells was determined by the methemoglobin method.[36] The concentration of GSH and GSSG was expressed in micromoles per gram of Hb.

Determination of GPx and GRd Activities

Aliquots of saline-washed red blood cells were thawed and hemolized (1:20) with 10 mM phosphate buffer, 1 mM EDTA-Na_2, pH 6.5, at 4°C for 5 minutes, and centrifuged at 20,000 × g for 15 minutes at 4°C. For GPx determination, 120 µl supernatant was incubated in a final volume of 3 ml with 100 mM phosphate buffer containing 1 mM EDTA-Na_2, pH 7.5, in the presence of 30 µl of 20 mM NADPH, 100 µl of 60 mM GSH, and 4 µl (1 international unit [IU]) GRd for 5 minutes at room temperature. A total of 100 µl of 36 mM cumene hydroperoxide solution was added, and GPx activity was measured following the oxidation of NADPH for 3 minutes at 340 nm[37] in a spectrophotometer.†† GRd activity was measured in 35 µl supernatant incubated in a final volume of 508.5 µl with 100 mM phosphate-EDTA-Na_2 buffer, pH 7.5, containing 2.5 mM GSSG for 5 minutes at room temperature. A total of 8.5 µl NADPH 12 mM was added, and NADPH oxidation was followed for 3 minutes at 340 nm[37] in an ultraviolet (UV) spectrophotometer.§§ In both cases, nonenzymatic NADPH oxidation was subtracted from the overall rate. The activity of both enzymes was expressed in micromoles per minute per gram Hb.

Statistical Analysis

All data are expressed as the mean ± SEM. One-way analysis of variance followed by the Student *t* test was used to compare the differences between groups. $P<0.05$ was considered to be statistically significant.

RESULTS

Plasma levels of LPO and NOx are shown in Figure 1. LPO levels in plasma increased highly significantly in dogs without postextraction treatment with aMT compared to the plasma samples collected before surgery (20.04 ± 0.89 versus 6.89 ± 0.73 nmol/ml, respectively; $P<0.0001$). Treatment with aMT reduced plasma LPO levels to control values ($P<0.0001$).

Plasma nitrite levels were also significantly increased in the vehicle-treated dogs compared to the preextraction levels (72.88 ± 5.39 versus 36.05 ± 3.465 nmol/ml, respectively; $P<0.0001$). The dogs that received aMT had significantly lower levels of nitrite compared to control preextraction rates (17.66 ± 2.31 versus 36.05 ± 3.465 nmol/ml, respectively; $P<0.0001$), and very significantly reduced values

Bioxytech LPO-568, Cayman Chemical, Ann Arbor, MI.
** Bio-Tek Power-Wave, Microplate Scanning Spectrophotometer, Bio-Tek Instruments, Winooski, VT.
†† Bio-Tek Instruments.
‡‡ Shimadzu Deutschland, Duisburg, Germany.
§§ Shimadzu Deutschland.

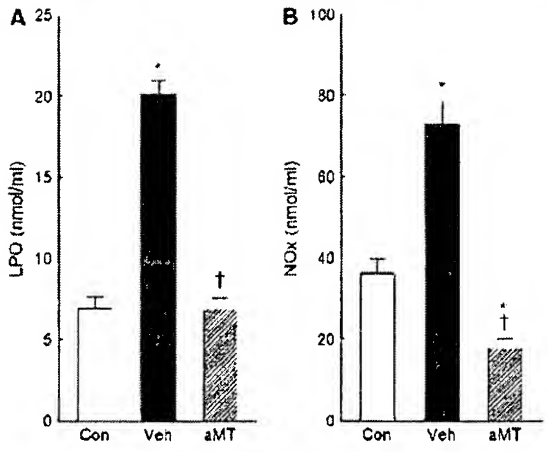

Figure 1.
*Plasma LPO **(A)** and NOx **(B)** levels in Beagle dogs before and after teeth removal and with and without aMT treatment. *P <0.0001 versus Con; †P <0.0001 versus Veh.*

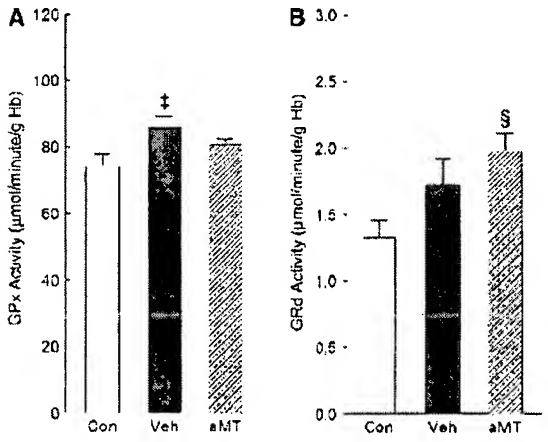

Figure 2
*GPx **(A)** and GRd **(B)** activities measured in red blood cells of Beagle dogs before and after teeth removal and with and without aMT treatment. ‡P <0.05 versus Con; §P <0.005 versus Veh.*

relative to those in dogs that received vehicle as the postextraction treatment (P <0.0001).

Figure 2 shows the activities of GPx and GRd in the preextraction and postextraction plasma samples. The vehicle-treated dogs after tooth extractions exhibited a significant increase in GPx compared to plasma samples collected before teeth extraction (85.82 ± 3.56 versus 74.38 ± 3.465 µmol/minute/g Hb, respectively; P <0.05); however, no significant change in GRd activity was measured (1.73 ± 0.19 versus 1.328 ± 0.13 µmol/minute/g Hb). Treatment with aMT significantly increased the activity of GRd, but not that of GPx, in dogs compared to the preextraction levels (1.98 ± 0.13 versus 1.328 ± 0.13 µmol/minute/g Hb, respectively; P<0.005).

Figure 3 summarizes the levels of total glutathione, GSH, GSSG, and GSSG/GSH ratio in red blood cells. Although there were no significant differences in the levels of total erythrocyte glutathione between animals of the three groups, tooth extractions without aMT treatment caused a significant rise in GSSG levels (2.36 ± 0.13 versus 1.69 ± 0.125 µmol/g Hb; P<0.005). The GSSG/GSH ratio also increased very significantly in erythrocytes of vehicle-treated dogs compared to the preextraction values (0.70 ± 0.03 versus 0.430 ± 0.04; P <0.0001). Treatment with aMT increased the levels of GSH significantly compared to values before teeth removal (3.93 ± 0.16 versus 4.47 ± 0.16 µmol/g Hb; P<0.05) and compared to the vehicle-treated dogs and to the control group without treatment (3.43 ± 0.22 versus 4.47 ± 0.16 µmol/g Hb; P<0.005). aMT also significantly reduced GSSG levels compared to those in vehicle-treated dogs (P <0.05); thus, the GSSG/GSH ratio was lower in aMT-treated animals (0.70 ± 0.03 versus 0.451 ± 0.02; P<0.0001).

DISCUSSION

Free radicals have been implicated in many pathophysiologic processes of the oral cavity.[2,6-15] The

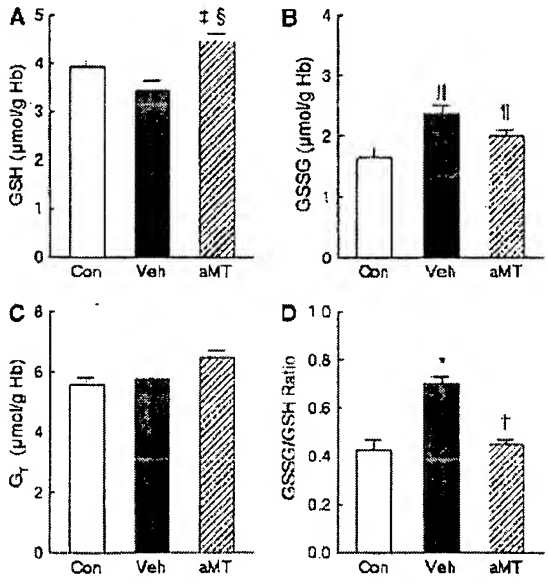

Figure 3.
*GSH **(A)**, GSSG **(B)**, and total glutathione **(C)** levels and the GSSG/GSH ratio **(D)** determined in red blood cells of Beagle dogs before and after teeth removal and with and without aMT treatment. G_T = glutathione. *P <0.0001, †P <0.05, and ‖P <0.005 versus Con; ‡P <0.0001, §P <0.005, and ¶P <0.05 versus Veh.*

relationships among aMT, the immune system, and oral status are not well defined. Reduced oral health (with advanced periodontal processes with gingival tissue damage and bone loss) serves as a trigger for increases in salivary aMT levels,[29,30] which in turn stimulates the CD4 lymphocytes.[30,31] However, there are no published reports related to aMT oxidative stress interactions during surgical oral interventions, including after tooth extractions. For the first time to our knowledge, the current results show the existence of significant oxidative stress during the immediate postoperative period following tooth extraction, with the changes being counteracted by local aMT application into the alveolar sockets after tooth removal.

One day after oral surgery, the dogs that did not receive aMT exhibited a significant increase in parameters of plasma oxidative stress as a consequence of the damage and the inflammatory process that follows surgical intervention. After tooth removal, bacteria of the oral cavity colonize the surface of the blood clot that covers the alveolar socket, granulation tissue, wound epithelium, and the adjacent gingival tissue.[15,38] These events cause an acute inflammatory response of the gingival mucosa, which surrounds the blood clot. Thus, during the first 24 to 48 hours postextraction, edema and vasodilatation are observed in the periphery of the alveolar socket with a marked infiltration of polymorphonuclear leukocytes.[15,38] Gingival tissue infiltration by polymorphonuclear leukocytes and monocytes, whose principal function after tooth extraction is phagocytosis of bacteria, is also responsible for the generation of ROS.[1,2,13,14] Besides the inflammatory process, other mechanisms, including breakdown of the gingival fibers, damage to periodontal vessels, and the mechanical mutilation to oral tissues as a consequence of tooth extraction, also participate in the oral damage after tooth removal.[15,38] Together, these events participate in promoting oxidative stress generated by the inflammatory process. In turn, the increased ROS stimulate the production of proinflammatory cytokines, transcription factors, such as nuclear factor-kappa B (NF-κB), and vascular cell adhesion molecules, thereby increasing the progression of the inflammatory process and the synthesis of RNS such as NO and peroxynitrite.[32,39]

Both ROS and RNS locally generated in the oral cavity after tooth removal can enter the circulation. In fact, plasma levels of LPO and NOx, which reflect the increased production of ROS and RNS, respectively, were significantly higher 24 hours after tooth extraction. Overproduction of lipid hydroperoxides and aldehyde products causes depletion of GSH, disrupting mucosal turnover.[1,9] Our results document these changes because GSSG levels and the GSSG/GSH ratio were significantly elevated in the vehicle-treated dogs. Thus, the observed differences in the cellular pool of GSH in the vehicle-treated animals reflect a generalized oxidative stress. These alterations may also produce changes in the GSH redox cycling enzymes. In fact, the increase in GPx activity after tooth extraction likely reflects the activation of the antioxidant machinery. However, the measured rise in GSSG was not adequately metabolized to GSH because of only a slight increase in GRd activity.

Recently, an inverse relationship between salivary aMT levels and periodontal status was found.[40] This study[40] supported a protective role of aMT against free radicals produced by inflammatory periodontal diseases. Herein, we found that the application of aMT into the alveolar sockets after tooth removal reduced significantly the oxidative stress parameters in both the plasma and the erythrocytes. Increased levels of LPO caused by tooth removal were counteracted by aMT at 24 hours after surgery. The ability of aMT to efficiently reduce the oxidation of lipids under a variety of conditions where free radicals are generated is well established.[20,21,29,41] It is likely that aMT achieves this high degree of lipid protection by neutralizing the radicals (i.e., hydroxyl radical and peroxynitrite) that initiate the process of lipid breakdown. aMT positions itself among the membrane lipids in such a way as to impede the oxidation of the polyunsaturated fatty acids.[42-44]

In the present study, aMT also counteracted NOx levels that were increased after oral surgery; in fact, the indole reduced NOx concentrations below those measured in plasma before tooth extraction. The effect of aMT on NOx levels may depend, at least in part, on its ability to scavenge nitrite.[41,45] Furthermore, in vivo studies have documented that aMT inhibits iNOS expression and activity in experimental models of sepsis in rats and mice.[28,29,40] Increased iNOS activity and expression are related to several oral mucosal inflammatory diseases;[4,5,7,11,12] thus, elevated iNOS activity probably contributes to the overproduction of NO and peroxynitrite during the inflammatory process following tooth removal. The inhibition of iNOS by aMT likely reduces NOx levels, thereby diminishing gingival damage and postextraction oxidative stress in the oral cavity. Additionally, aMT could also decrease nitrosative stress in gingival cells by directly neutralizing peroxynitrite.[45]

Besides reductions in plasma markers of oxidative (LPO) and nitrosative (NOx) damage, aMT also reduced significantly the GSSG/GSH ratio, the best index of intracellular oxidative damage in erythrocytes. In addition to the direct scavenging activity of aMT, which reduces GSH consumption,[26] aMT also increased GRd activity, which may account for the reduction of GSSG and increase of GSH levels, thereby providing the cell with additional GSH.[23,26]

Besides protecting GRd per se from oxidative destruction, the effect of aMT on GRd activity also may depend on a genomic effect of the indolamine to increase the expression of the enzyme.[24,25] Regulation of the GSH redox cycling is probably of great significance for oral tissue homeostasis, because GSH is a major endogenous antioxidant in the cell. GSH plays an important role in cellular protection from oxidative damage of lipids, proteins, and nucleic acids.[46] Additionally, GSH regulates the metabolism and activity of other proteins and it interacts synergistically with other components of the antioxidant defense system, such as vitamins C and E and superoxide dismutase.[47,48]

Although these data support the ability of aMT to reduce oral surgery–dependent oxidative stress, the two-faced character of ROS-RNS should be noted.[49] Although overproduction of ROS-RNS should be considered a protective response of the immune system to prevent bacteria infection, it also results in oxidative stress and cell damage. By contrast, beneficial effects of these radicals occur at low to moderate concentrations, and involve physiologic roles in a number of cellular signaling pathways.[49] Thus, the organism tends to prevent an excess of ROS-RNS activating the antioxidative response. In this regard, exogenous administration of aMT should not be considered a treatment to minimize ROS-RNS. Instead, aMT may help in preventing the overproduction of free radicals by maintaining their basal levels. Whether the effects of aMT reported here favor healing reaction is yet unclear and requires further research.

CONCLUSIONS

Our results document a significant increase in the levels of oxidative stress, as measured by blood parameters, in the immediate postoperative period following tooth removal. These results suggest that the use of antioxidants, such as aMT, may be a beneficial therapy after surgical procedures in the oral cavity.[50] Locally, administration of aMT into the alveolar sockets successively counteracted oxidative and nitrosative stress in blood, presumably also reflecting the reduction of damage in the oral cavity. Overproduction of ROS-RNS in gingival cells after tooth extraction contributes to postextraction inflammatory and infectious complications. The subsequent increase in the damage to oral tissues may, in turn, delay postextraction wound healing and regeneration of gingival tissue surrounding the alveolar bone. Thus, increased oxidative stress after tooth removal would be related to a poor postextraction prognosis and evolution, mainly when a concomitant inflammatory disease is present in the oral cavity.[40] Because the antioxidant defense system in the oral cavity counteracts this oxidative stress, stimulation of this defense system aids in wound healing and reduces the recovery. Patients with compromised antioxidant defenses in the oral cavity or with pathologies associated with oxidative stress, such as diabetes, Parkinson's disease, autoimmune disorders, periodontal disease, or aphthous ulceration, have elevated levels of ROS-RNS, which aggravates the damage to gingival tissue, delaying the regeneration processes. The current results suggest that local application of aMT may be useful in preventing inflammatory and infectious complications induced by oxidative stress after tooth extraction.

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